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GRANT NUMBER DAMD17-96-1-6048

TITLE: Role of a Placenta-Specific Gene in Mammary Tumorigenesis

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED	
	August 1998	Annual (1 Jul 97 - 1 Jul 98)	
4. TITLE AND SUBTITLE Role of a Placenta-Specific Gene in Mammary Tumorigenesis		5. FUNDING NUMBERS DAMD17-96-1-6048	
6. AUTHOR(S) Joshua N. VanHouten			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Health Research, Incorporated Roswell Park Cancer Division Buffalo, New York 14263		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT <i>(Maximum 200)</i> We have previously shown that mouse mammary preneoplasias and carcinomas of several etiologies ectopically express 2.2 and 5.6 kb MIPP-related mRNAs. This raises the possibility that MIPP might be a mouse mammary oncogene. Furthermore, MIPP is assigned to the actin-binding <i>kelch</i> family based on sequence analysis. However, the product(s) of the MIPP gene has not yet been identified. Therefore, the purpose of this research is to clone the 2.2 and 5.6 kb RNAs for use in transformation assays and functional studies. The 5' end of the 2.2 kb transcript was cloned by RACE using the SMART oligonucleotide (Clontech). The cDNA contained a 1.7kb open reading frame coding for 556 amino acids. Sequence analysis revealed the presence of an N-terminal BTB protein/protein interaction domain plus six <i>kelch</i> repeats at the C-terminus. An epitope-tagged recombinant MIPP protein is being expressed in EL12 mouse mammary epithelial cells to determine 1) whether they become transformed, and 2) its subcellular location and interaction with actin. Efforts are also focused on cloning the 5.6 kb mammary carcinoma-specific transcript. This work could lead to a better picture of how derangement of the actin cytoskeleton relates to mammary tumorigenesis.			
14. SUBJECT TERMS Breast Cancer MIPP, retrotransposon, mammary epithelial cells, mouse model		15. NUMBER OF PAGES 18	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

19981029024

FOREWORD

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INTRODUCTION:

The mouse IPP gene (MIPP) contains a long terminal repeat (LTR) from an intracisternal A-particle (IAP), an endogenous retrovirus-like element. The solo IAP LTR present in the MIPP gene promotes placenta-specific expression of a 1.2 kb message. This transcript is a truncated form of 2.2 and 4.4 kb MIPP transcripts also found in placental tissue (1). The MIPP gene appears to have an important function because it is evolutionally conserved. Sequences homologous to MIPP were detected in monkey, hamster, and human DNA by Southern blotting (2). The human homolog of MIPP, human IPP (HIPP), was assigned to chromosome 1p32-1p22 (3). The truncated 1.2 kb MIPP transcript codes for a putative protein of 202 amino acids, and has four repeated segments (*kelch* repeats) found in members of the *kelch* family of proteins (4). Several *kelch* family proteins have been shown to bind actin. Most of these proteins have an N-terminal protein/protein interaction domain called BTB in addition to the *kelch* repeats. The MIPP product has only been deduced from nucleic acid sequences and has not been directly identified or characterized to date.

We have previously shown that BALB/c mouse mammary preneoplasias and carcinomas of several etiologies ectopically express 2.2 and 5.6 kb MIPP-related mRNAs. The 5.6 kb transcript is unique to the mammary lesions. MIPP messages were not detected in normal mammary gland from virgin, pregnant, and lactating mice. Additionally, in the mouse mammary tissues, IAP expression was found to correlate with MIPP expression. However, we showed that neither the 2.2 kb nor the 5.6 kb RNAs were promoted by the MIPP gene's LTR. Furthermore, we determined that these MIPP transcripts share a common 3' end with the 1.2 kb mRNA. We were unable to detect expression of HIPP in human breast cancer cell lines using RT-PCR.

The ectopic expression of MIPP in the mammary tumors makes it a candidate for a novel oncogene in BALB/c mice. Therefore, one of the purposes of the proposed research is to determine whether the two MIPP mRNA species expressed in BALB/c mammary carcinomas are oncogenic. This necessitates cloning these transcripts. Using a degenerate primer derived from conserved regions of the BTB domain, we previously cloned a segment of the 5' end of the 2.2 kb transcript. Its reading frame was continuous with that of the 1.2 kb transcript, and coded for a BTB domain plus six C-terminal *kelch* repeats in total. Still, the entire 5' end of the 2.2 kb transcript, as well as the 5.6 kb transcript, remained unknown. Accordingly, a goal of this research is to obtain the rest of the MIPP cDNA sequences and to transfet them into mouse mammary epithelial cells for growth/tumorigenicity/invasiveness assays. Use of MIPP cDNAs in an expression system will also be of value in isolating and determining the function of MIPP protein(s).

EXPERIMENTAL METHODS, ASSUMPTIONS, AND PROCEDURES:

1. Toward the aim of cloning the MIPP mRNAs, the following strategies were used.

A. First, a cDNA library was made from a MIPP-expressing tumor using the SMART cDNA library synthesis kit (Clontech). The SMART oligonucleotide recognizes the 7-methylguanosine cap at the 5' end of eukaryotic mRNAs. When the reverse transcriptase (RT) reaches the 5' end of the mRNA, it switches templates to copy the SMART oligonucleotide. Long distance PCR is then used to amplify second strand cDNA. The result is a cDNA library enriched for full-length clones. After the long distance PCR (Figure 1), the cDNA was end-polished, ligated to adapters, and size

selected for >500bp on a Sephadex column. The cDNA was then ligated into λgt11, packaged, and titered. One million plaque forming units (pfu) were screened with a ³²P-labelled MIPP cDNA probe. The library was also screened using PCR with primers that amplify a 250bp fragment of the 3' end of the MIPP RNAs. PCR was also performed on the first and second strand cDNA to verify the presence of MIPP sequences (Figure 2).

B. A variation of standard 5' RACE was used to clone the 2.2 kb MIPP transcript from a mouse mammary tumor. A MIPP-specific oligonucleotide was used to prime reverse transcription of total RNA, and the SMART oligonucleotide was included in the RT reaction. Therefore, no further modification of the first strand cDNA was necessary. Nested MIPP-specific primers were used with the SMART-specific primer in long distance PCR. To clone the 5.6 kb RNA's 5' end, the same RACE technique was done with the addition of GC-melt reagent (Clontech). Resulting bands were cloned into T-overhang plasmid vectors and sequenced. One cDNA clone representing the 5' end of the 2.2 kb MIPP transcript was isolated and used to probe a Northern blot of total RNA from a D2 mouse mammary tumor (Figure 3).

2. The following steps have been taken toward expression of the open reading frame of the 2.2 kb MIPP transcript in normal mouse mammary epithelial cells: A) epitope-tagging and subcloning of the open reading frame, B) optimization of selective media and transfection conditions, C) analysis of mixed pools and single clones of transfectants for recombinant MIPP RNA expression.

A. The 1.7 kb open reading frame (orf) of the 2.2 kb MIPP RNA was amplified using a high-fidelity PCR kit (Boehringer-Mannheim). A Kozak mammalian consensus translational initiation sequence, a His₆ epitope tag, and a BamHI site to facilitate cloning were incorporated into the 5' PCR primer. The 3' primer contained an EcoRI site after the MIPP stop codon. The PCR product was purified from an agarose gel, digested with BamHI and EcoRI, and ligated into the BamHI/EcoRI site of the mammalian expression vector pCDNA3.1 (Figure 4). This construct was sequenced to confirm that the epitope tag and the MIPP sequences were in frame and that there were no stop codons.

B. The conditions were optimized for selection and transfection of the mouse mammary epithelial cell line EL12 (5). A kill curve for EL12 cells was made by growing the cells in 60mm dishes with 0, 100, 200, 400, 600, or 800 µg/ml of geneticin (G418). The cells were grown for 5 days, and the medium was changed on days 2 and 4. On day 5, the relative number of cells was measured by eye and by reading the absorbance at 800nm. A concentration of 500mg/ml was chosen based on the kill curve (Figure 5). To optimize the conditions for transfection into EL12 cells, the luciferase assay (Promega) was used. TransFast reagent (Promega) was used to transfet the cells with 1, 2, or 4 µg of pGL3 luciferase plasmid (Promega) at a ratio of 1:1 or 2:1 TransFast:DNA. Three replicates were done for each condition in 35mm culture dishes. The best results were obtained with 4µg DNA per 35mm dish at a 1:1 ratio of TransFast reagent:DNA (Figure 6).

C. The optimal transfection conditions were scaled-up to a 100mm dish format, and EL12 cells at 75% confluence were transfected with either pCDNA3.1(MIPP) or empty vector. After 48 hours in non-selective media, the cells were replated with G418 at densities ranging from 1:5 to 1:1000. Four individual colonies of the EL12 cells transfected with pCDNA3.1(MIPP) and one colony of the control transfection were

isolated by scraping and aspirating with a glass pipet. Total RNA was prepared using TRI reagent (MRC) and protein was isolated by boiling the cell pellet in SDS sample buffer. Some of each RNA sample was subjected to DNaseI digestion prior to reverse transcription using an oligo-dT primer. PCR was then performed with two sets of primers: one to amplify the 1.7 kb recombinant orf only, and one to amplify a 250bp fragment extending beyond the 3' end of the orf (Figure 7). The former was done to assay recombinant MIPP expression, and the latter to assay endogenous MIPP expression. Northern and Southern blotting with a MIPP probe and Western blotting with an anti-His₆ antibody (Boehringer-Mannheim) are in progress.

RESULTS AND DISCUSSION:

1. A. The high frequency of ectopic MIPP expression in both mouse mammary preneoplasias and tumors suggests that MIPP might be a new oncogene which contributes to at least some pathways of neoplastic progression in the BALB/c mouse mammary gland. To determine whether MIPP is a mammary oncogene, it is necessary to clone and characterize the transcripts expressed in the tumors. The effects of these clones on the *in vitro* and *in vivo* growth properties of mammary epithelial cells can then be analyzed. We have previously cloned part of the 5' end of the 2.2 kb transcript, but the MIPP 5' sequences are not completely known. Therefore, a cDNA library was made from a MIPP expressing tumor using the SMART cDNA library synthesis kit (Clontech). The SMART oligonucleotide recognizes the 7-methylguanosine cap at the 5' end of eukaryotic mRNAs. When the reverse transcriptase reaches the 5' end of the mRNA, it switches templates to copy the SMART oligonucleotide. Long distance PCR is then used to amplify second strand cDNA. The result is a cDNA library enriched for full-length clones. The library was screened with a MIPP nucleic acid probe and by PCR, but no positive clones were identified.

B. Using the SMART oligonucleotide in a modified version of 5' RACE, the 5' end of the 2.2 kb MIPP transcript was amplified from a mouse mammary tumor's RNA. Figure 8 is the translation of the longest open reading frame of the 2.2 kb transcript. The cDNA has an open reading frame of about 1.7kb that potentially codes for a 556 amino acid protein with a theoretical MW of 62kDa. ProfileScan (<http://ulrec3.unil.ch/>) was used to search for known domains in the putative MIPP protein. The highest scoring match was to the BTB domain at position 28-118 (normalized score 20.29). A probe from the 5' end of the 2.2 kb transcript (including the BTB domain) hybridized to 2.2, 3.5, and 5.6 kb RNAs from a D2 mouse mammary tumor. The 3.5 kb species may be from a related gene which contains a BTB domain but not *kelch* repeats since it is not detected with a probe from the 3' end of MIPP transcripts (6). Many such genes are identified when BLAST is used to search GenBank against the 2.2kb cDNA sequence.

Several sets of primers and PCR conditions were tried before the RACE technique worked. The successful conditions involved uneven annealing temperatures in the first round of PCR, and the use of cDNA primed with a MIPP-specific oligonucleotide. These experiments underscore the difficulty encountered in cloning the 5' ends of MIPP transcripts, and raise the possibility that they possess some property that is resistant to reverse transcription and/or PCR. One possible problem might be high GC-content, but even when the RACE was specifically tailored for GC-rich sequences, no products were obtained. Thus, the 5' end of the 2.2kb cDNA, but not the 5.6kb cDNA, has been cloned.

2. Since it has six *kelch* repeats and a BTB domain, the putative MIPP protein is a member of the *kelch* family. The *kelch* family includes: the *Limulus* protein, scruin, the *Drosophila* *kelch* gene product, various vaccinia virus proteins, mammalian calicin, the nerve-cell-specific ENC1, and the *Caenorhabditis elegans* protein spe26 (7). Scruin is an actin filament cross-linking protein in *Limulus* sperm (8), kelch associates with actin in structures known as ring canals during oogenesis in *Drosophila* (9), calicin is associated with actin in the acrosome of mammalian sperm (10), and ENC1 is an actin-binding protein found in nervous cells (11). Furthermore, it has been suggested that the vaccinia proteins in this family may mediate the effects of poxviruses on the host cells' actin cytoskeleton to facilitate their intracellular movement and infectivity (8; 12).

The actin cytoskeleton serves various functions, some of which are directly altered in the malignant cell phenotype (reviewed in 13). The actin filament network functions in determining cell shape and movement, mediates cytokinesis, and participates in cell-cell interactions and signal transduction pathways via associations with plasma membrane proteins. Disruption of these functions correlates closely with derangements in growth behavior and morphology that characterize malignantly transformed cells. Indeed, disorganization of the actin cytoskeleton itself is often an attribute of transformed cells.

It is our hypothesis that the MIPP protein's function involves binding to actin, thereby contributing to neoplastic progression when ectopically expressed. To address this question, the epitope-tagged 1.7kb MIPP orf was transfected into the EL12 mouse mammary epithelial cell line. TransFast (Promega), a cationic lipid reagent, was used, and conditions were optimized with a luciferase assay. Single colonies of cells were isolated and screened for recombinant and endogenous MIPP expression by RT-PCR. Two out of four clones expressed the recombinant MIPP 1.7kb orf, but one of these also had a band in the endogenous MIPP reaction (Figure 7). This induction of endogenous MIPP expression could be a positional effect, since it was seen in only one clone and not in the control-transfected cells. Southern blotting should reveal whether this is the case. Northern blotting is being done to confirm RT-PCR results, and Western blotting will identify the protein product. Future experiments include transformation assays and immunoprecipitation. If our hypothesis is correct, the transfected cells should acquire more properties associated with malignant transformation, and actin should co-immunoprecipitate with the anti-His₆ antibody.

Through immobilized metal affinity chromatography, the His₆ tag will also allow highly purified protein to be produced, which can then be used to raise antisera against MIPP protein(s). This may provide an alternative method to clone the 5.6kb cDNA. If the 2.2 and 5.6 kb mRNAs encode different proteins, since their coding sequences overlap, both could be immunoprecipitated with the antiserum mentioned above. The larger protein product of the 5.6kb RNA could then be sequenced at its N-terminus. Degenerate oligonucleotide primers from the N-terminus could then be used in specific PCR reactions with downstream MIPP primers. One drawback of this method is that it would not detect a 5' untranslated region.

RECOMMENDATIONS RELATED TO STATEMENT OF WORK (SOW):

Task number one, which was essentially to determine whether the LTR promotes MIPP transcription in the mouse mammary tumors, has previously been accomplished. Tasks two and three are to determine whether MIPP is an oncogene in mouse mammary carcinogenesis and to determine the function of MIPP protein(s), respectively. These tasks depend upon the cloning of the tumor-associated MIPP transcripts. Thus far, the sequence of the 2.2kb cDNA has been determined, and experiments related to its protein product's function and oncogenicity are underway. Future research will continue to concentrate on new ways to clone the 5' end of the 5.6kb MIPP RNA. Progress has been made previously on task four, analyzing HIPP in human breast cancers. HIPP expression was not detected in human breast cancer cell lines. However, analysis of breast cancer tissues for HIPP expression and analysis of the HIPP gene in DNA of breast cancers and breast cancer cell lines will still be done. These experiments are warranted because genes are frequently differentially expressed in cultured cancer cells and cancer tissues.

CONCLUSIONS:

We have cloned the 2.2kb MIPP transcript from a BALB/c mouse mammary tumor. It contains a 1.7kb orf capable of coding for a 556 amino acid protein with an N-terminal BTB domain and six C-terminal *kelch* repeats. A His₆ epitope tag was added to the orf and it was cloned into an expression vector. Conditions for transfection and selection of EL12 mouse mammary epithelial cells were optimized before the cells were transfected with the 1.7kb orf. Individual colonies of cells were obtained, at least one of which expresses the recombinant MIPP orf as detected by RT-PCR. Experiments to confirm expression of the recombinant MIPP RNA and protein are in progress. When they are complete, the more significant experiments of determining the functional and oncogenic properties of MIPP will be possible. These experiments have the potential to provide a unique perspective on derangement of the microfilament network in mammary tumorigenesis.

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FIGURE LEGENDS:

Figure 1

Second strand cDNA made from total RNA with the SMART cDNA synthesis kit (Clontech) was separated on an agarose gel and stained with ethidium bromide. The samples were liver, the positive control provided in the kit, and a D2 mouse mammary tumor previously shown to express MIPP. Between the two samples is the 1000bp ladder (Gibco-BRL).

Figure 2

PCR was done on first and second strand cDNA and dilutions of a SMART cDNA library from a D2 tumor to amplify a 250bp fragment from the 3' end of MIPP. Both cDNAs contain the MIPP sequences, but the library dilutions look identical to the water control (not shown).

Figure 3

The first 900bp of the 2.2kb MIPP cDNA were used to probe a Northern blot of RNA from a D2 mouse mammary tumor (A). Panel B shows ethidium bromide staining of the Northern, done to assess RNA quality.

Figure 4

A graphical representation of the pCDNA3.1(MIPP) construct showing the 1.7kb orf, the His6 epitope tag, and the BamHI (B) and EcoRI (E) sites is shown at the top. Below is a BamHI/EcoRI digestion of the purified construct on an ethidium bromide-stained agarose gel.

Figure 5

EL12 cells were plated at the same density on 60mm dishes. The growth medium was supplemented with the indicated amounts of G418. After 5 days, the confluence of the cells was estimated by eye (visual). Subsequently, they were detached, resuspended, and the optical density at 800nm (OD800) then measured in a spectrophotometer.

Figure 6

The plasmid pGL3 was transfected into EL12 cells under varying concentrations of DNA and TransFast reagent, and luciferase activity was measured as relative light units (RLU) in a luminometer. The conditions were 1:1 TransFast:DNA (1, 2, 3) or 2:1 TransFast:DNA (4, 5, 6) and 1 μ g DNA (1, 4), 2 μ g DNA (2, 5), or 4 μ g DNA (3, 6) per 35mm dish. Three replicates were done of each condition.

Figure 7

Single colonies of EL12 cells transfected with the 1.7kb MIPP orf (H1, H2, H3, and H5) or with empty vector (C2) were analyzed for recombinant (1700) and endogenous (250) MIPP expression by RT-PCR. A reaction was done with no template for a negative control (water), and for the 250bp fragment, the original MIPP cDNA was included as a positive control (+).

Figure 8

Translation of the 1.7kb orf from the 2.2kb MIPP cDNA. The BTB domain is bold and underlined, and the *kelch* repeats are double-underlined.

FIGURE 1

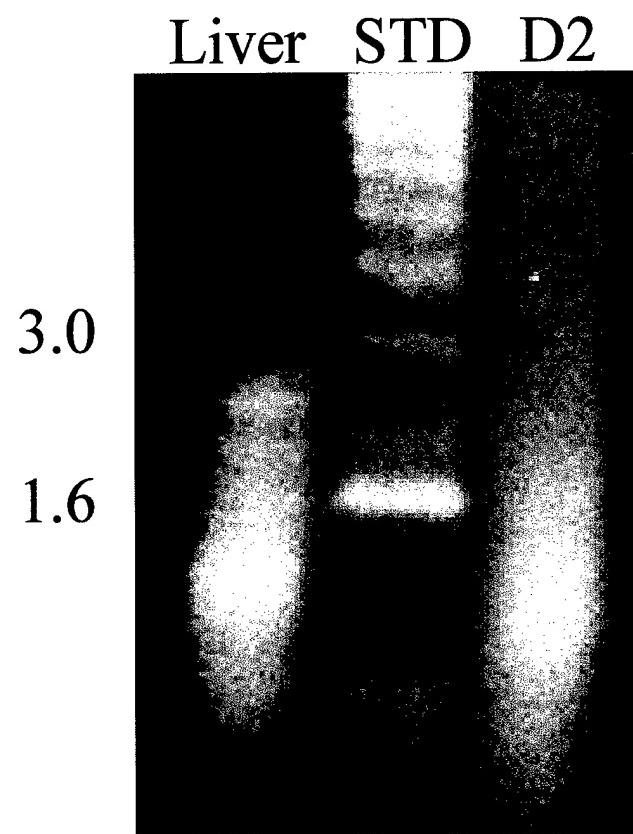


FIGURE 2

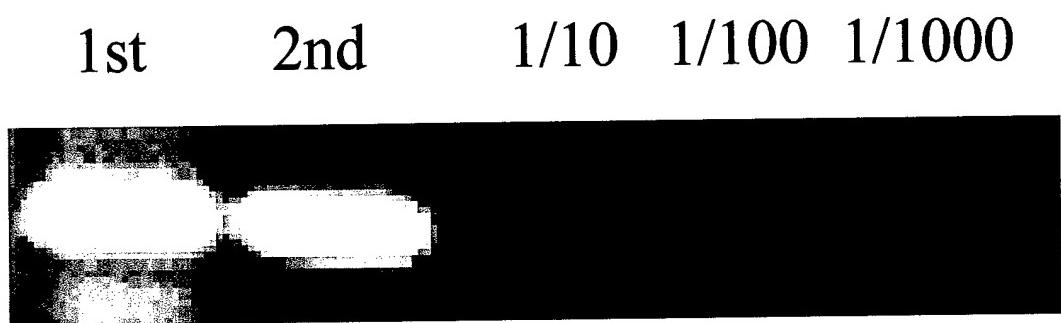


FIGURE 3

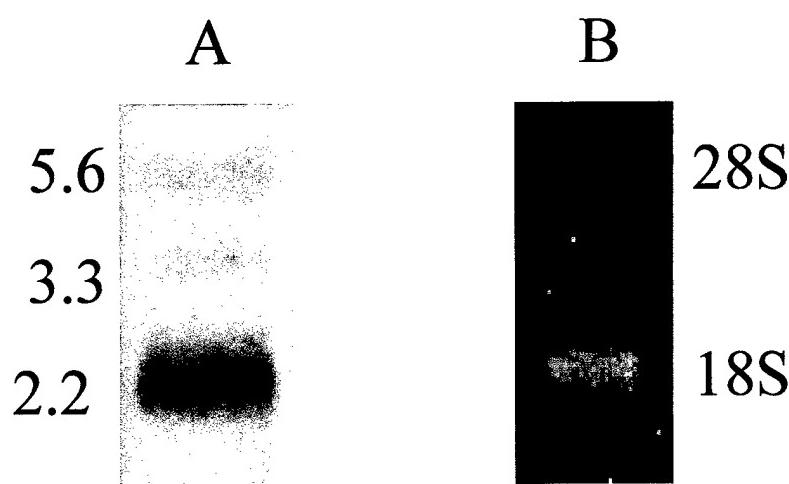


FIGURE 4

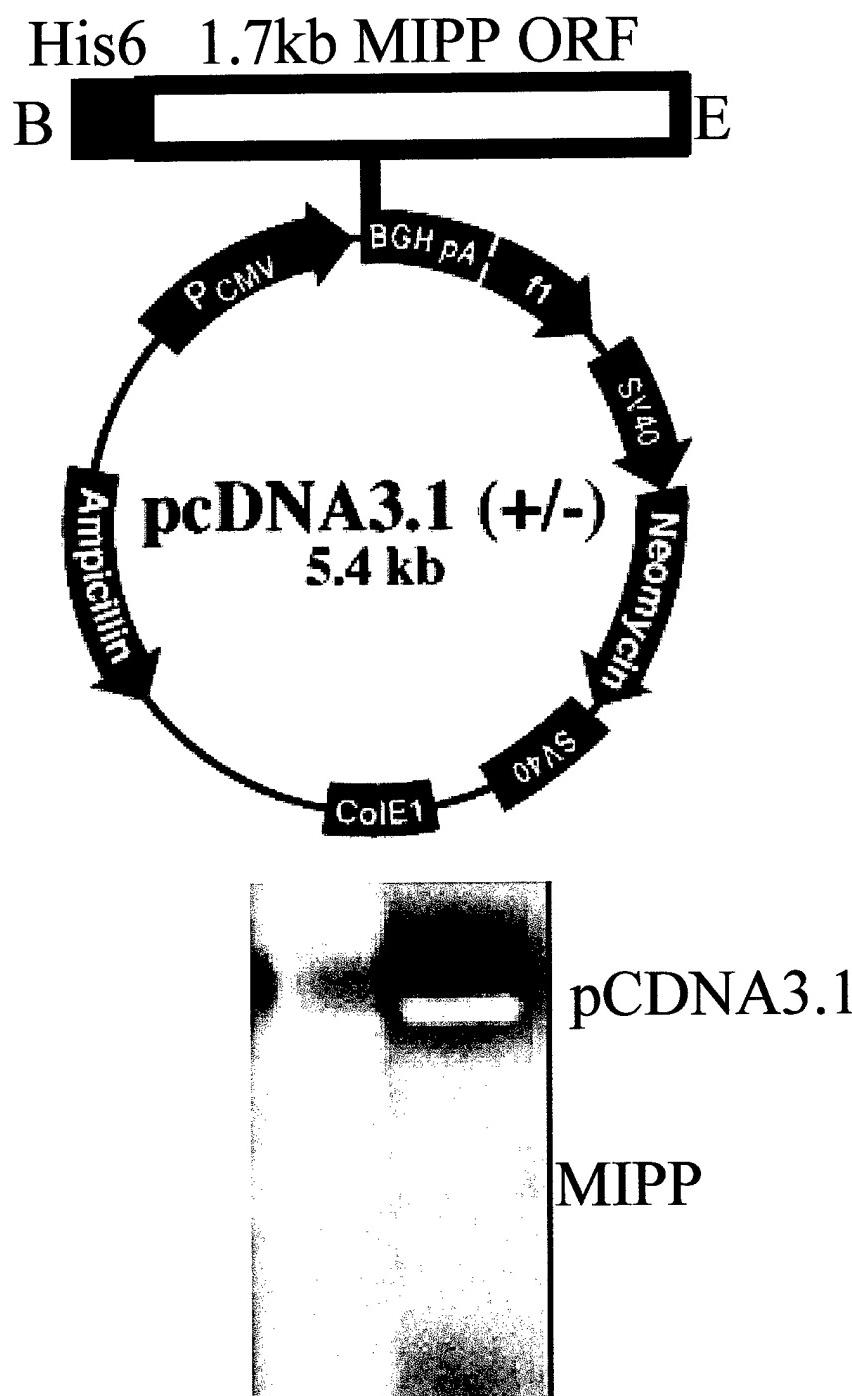


FIGURE 5

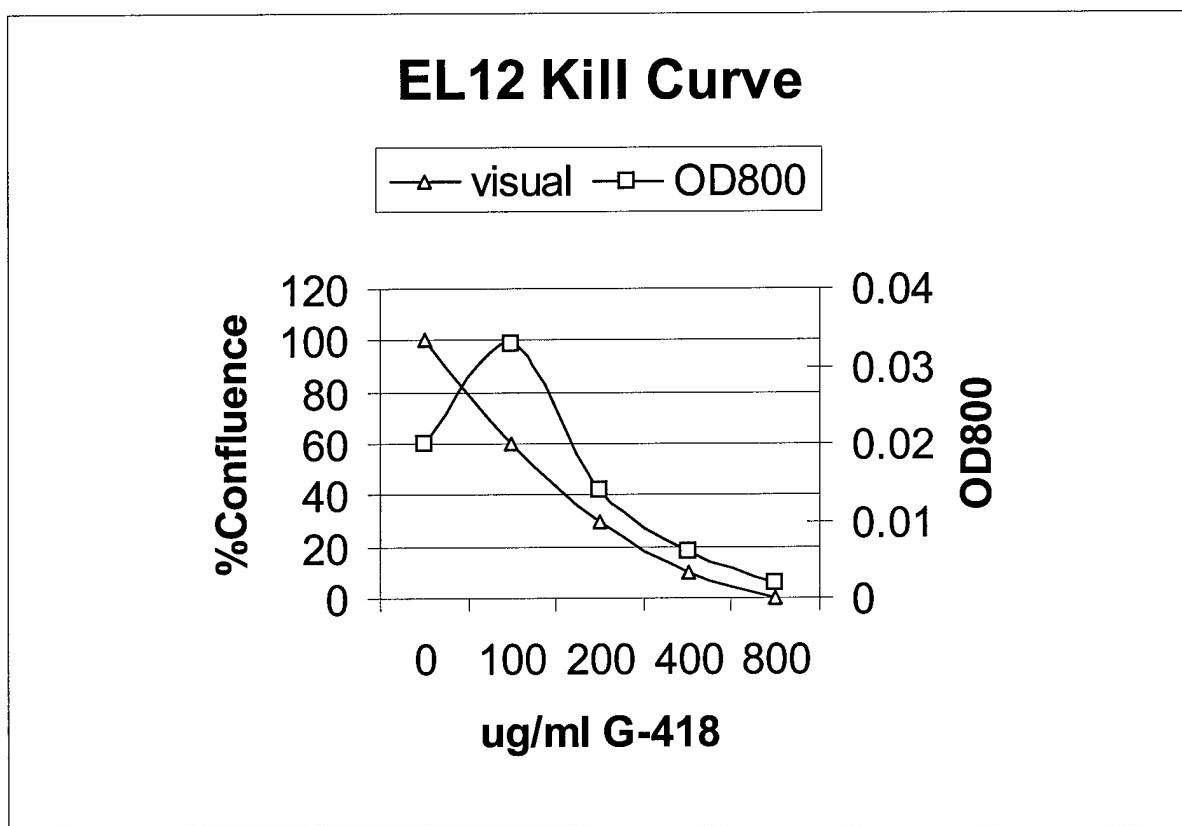


FIGURE 6

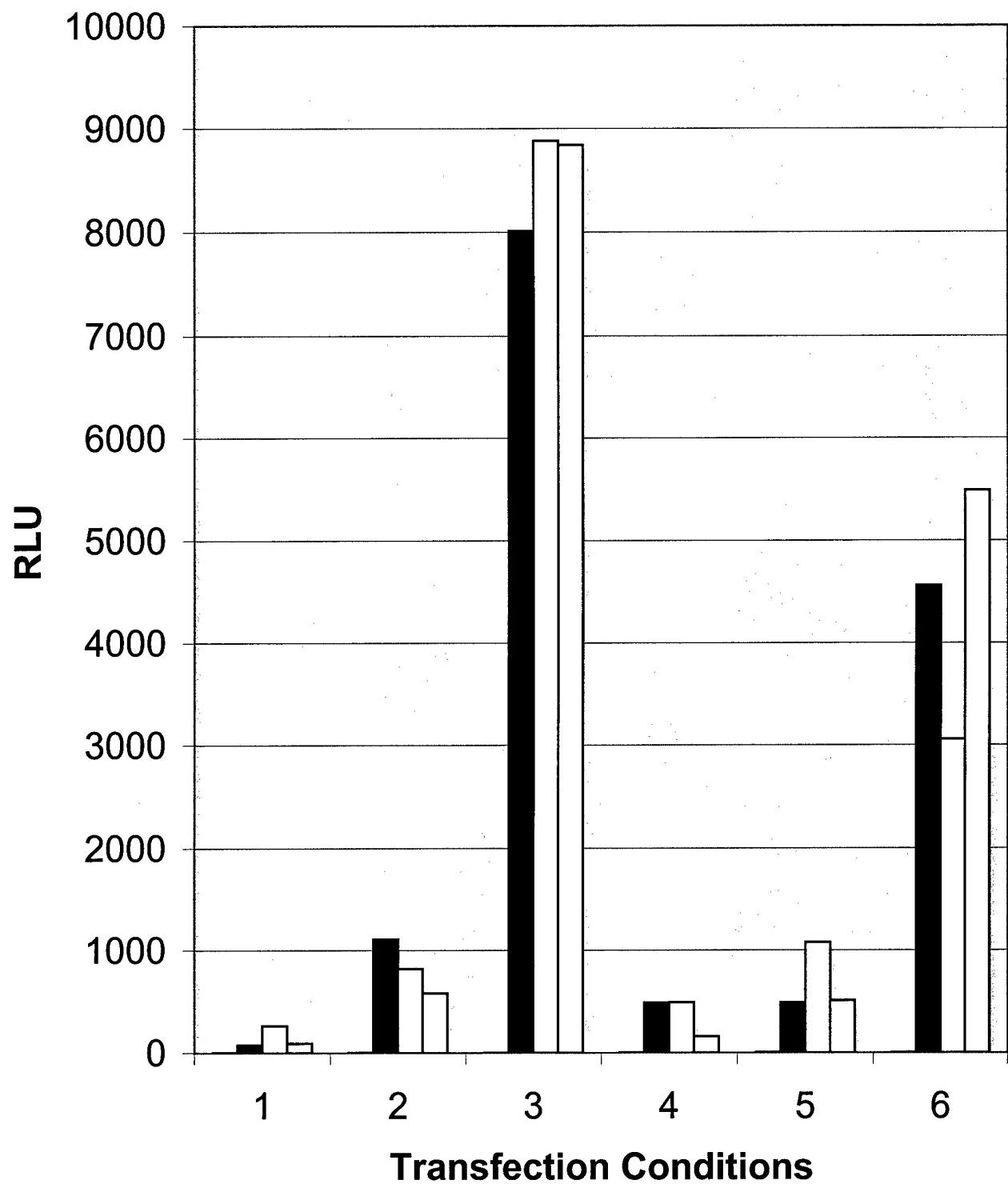


FIGURE 7

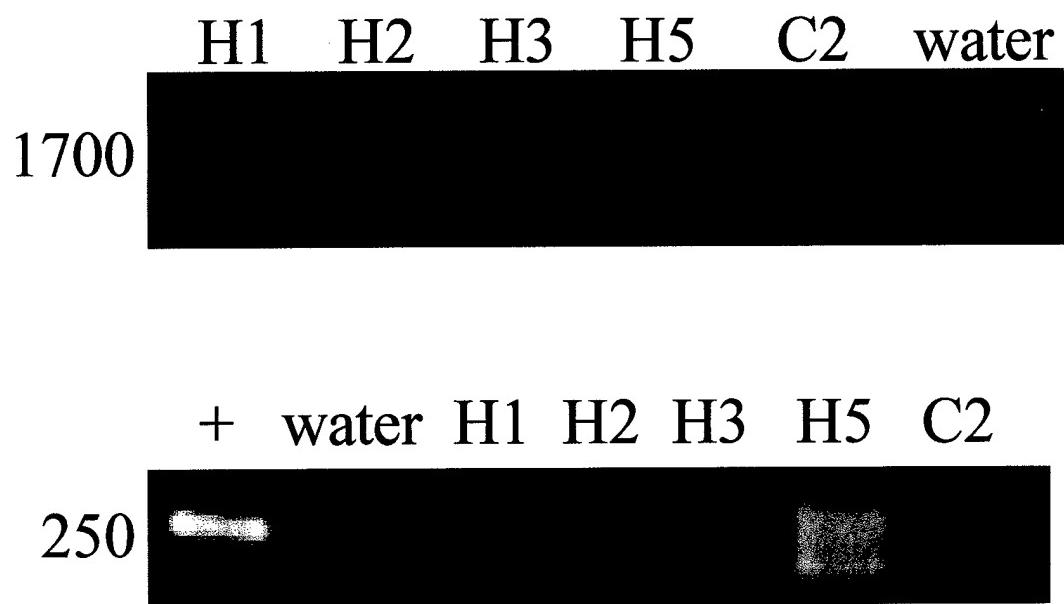


FIGURE 8

MSKEEYAKAADSSFSSDKHAQLILAQMNMRTGQHFCDVQLQVG
KETFQVHRLVLAASKPYFAALFTGGMKESSKDVVQILGVEAGIF
QLLDFIYTGVVNIAVTNVQELIVAADMLQLTEVVNLCCDFLKGQ
IDPQNCIGLFQFSEQIACHDLLEFTENYIHSIFLEVHTGEEFLGLTKDQ
LIKILRSEELSIEDEYQVFLAAMQWILKDLGKRRKHVVVEVLDPVRFPL
LPSQRLLKYIEGVSDFNLRVALQTLKEYCEVCKSPKENKFCSFLQTS
KVRPRKKARKYLYAVGGYTRLQGGRWSDSRALSCVERFDTSQYW
TTVSSLHQARCGLGVAVVGGMVYAIGGEKDSMIFDCTECYDPVTQ
WTTVASMNHPRCGLGVCVCYGAIYALGGWVGAEIGNTIERFDPDEN
KWEVVGSMAVSRYYFGCCEMQGLIYAVGGISNEGLELSFEVYDPL
SKRWSPPLPPMGTRRAYLGVAALNDIYAIGGWNETQDALHTVEKYS
FEEEKWVEVASMKVPRAGMCAVTVNGLLYVSGGRSSSHDFLAPGT
FGLS*